

Report

Cleavage of MCM2 licensing protein fosters senescence in human keratinocytes

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In eukaryotic cells, MCM, the minichromosome maintenance proteins, form a heterohexamer during G₁ phase in the cell cycle and constitute a DNA helicase activity at the onset of replication. MCM proteins are downregulated and dissociated from chromatin when cells exit the cell cycle. MCM proteins are upregulated frequently in a variety of dysplastic and cancer cells. To delineate the role of MCM in esophageal epithelial biology, we determined the MCM family gene expression during cellular senescence, immortalization, differentiation and apoptosis. All of the MCM2-7 proteins appeared to be downregulated in primary human esophageal keratinocytes upon replicative senescence. Their expression was restored by ectopic expression of a catalytic subunit of human telomerase, resulting in immortalization. Interestingly, we found a reciprocal induction of a novel MCM2-related protein fragment upon cell growth inhibition associated with senescence, contact inhibition or terminal differentiation, but not apoptosis. Epitope mapping of this MCM2-related fragment suggested the lack of amino- and carboxyl-terminal regions, including one of the putative nuclear localization signals and the ATPase domain, the MCM box. The absence of multiple MCM2 transcripts implied a possible posttranslational molecular cleavage in generation of the MCM2-related fragment, and a potential functional role in the regulation of the activity of the MCM protein complex.

Introduction

DNA replication occurs in a precise fashion during eukaryotic cell division. This tight control is orchestrated by many regulatory molecules, including members of the minichromosome maintenance gene family, designated as MCM. Initially, MCM proteins are recruited to sites of DNA replication and interact with each other, forming the MCM2-7 complex during G₁ phase.¹ This complex has

helicase activity and facilitates DNA replication.^{2,3} Therefore, the MCM proteins are essential for proliferating cells. In fact, MCM proteins are upregulated frequently in a variety of dysplastic and cancer cells.⁴⁻⁶

Normal human epithelial cells are limited in their proliferative capacity and eventually undergo differentiation, senescence or apoptosis. In this context, deregulated cells have mechanisms to suppress such processes, thereby resulting in unlimited cell proliferation, termed immortalization. Immortalization of human esophageal epithelial cells can be achieved by the ectopic expression of human telomerase, hTERT.⁷ Importantly, these cells maintain cell cycle checkpoints such as p16^{INK4a}/pRb and p14^{ARF}/p53/p21^{WAF1}.⁷ When oncogenic Ha-Ras is expressed ectopically in immortalized human esophageal epithelial cells, these cells undergo senescence, accompanied by upregulation of p16^{INK4a} and hypophosphorylated pRb.⁸ To understand the molecular mechanisms underlying constrained cellular growth arrest induced by senescence or differentiation, we used normal human esophageal epithelial cells (EPC2) and their derivative immortalized cells (EPC2-hTERT). We found that MCM2 was cleaved in senescence, while cancer cells prevented MCM2 from being cleaved. These results might indicate that the cleavage of MCM2 plays a critical role in cellular growth arrest induced by senescence or differentiation.

Results

A novel MCM2-related protein fragment is induced upon replicative senescence through a post transcriptional mechanism. We have previously carried out extensive characterization of EPC2 primary normal human esophageal epithelial cells.⁷ EPC2 cells cease proliferation as they undergo replicative senescence by 44 PD with an induction of p16^{INK4a} protein and senescence-associated β -galactosidase activity. Retrovirus-mediated stable transduction of a catalytic subunit of telomerase (hTERT) in the presenescent EPC2 cells (42 PD) permitted the cells to reenter the cell cycle and resulted in immortalization.⁷ The MCM family proteins are associated with cell proliferation.⁹ In agreement, all of the examined MCM family members were found downregulated as EPC2 cells underwent senescence as documented by a reduced phosphorylation level of pRB protein (Fig. 2A). By contrast, their expression was reversed

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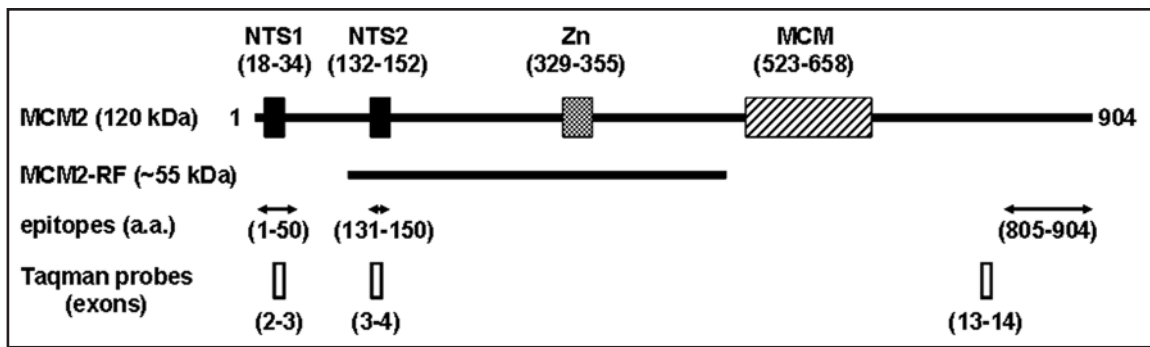


Figure 1. Schematic representation of the MCM2 protein and a novel MCM2-related fragment. Horizontal bars indicate the primary structure of the 120 kDa MCM2 protein and putative molecular mass of approximately 55 kDa (MCM2-RF), sharing an epitope recognized by an antibody directed against amino acids 131–150 of human MCM2. Failure of detection of the MCM2-RF by antibodies directed against peptides containing amino acids 1–50 or amino acids 805–904 (see Figs. 3–5), indicated by horizontal arrow, predicted the molecular region of MCM2-RF. MCM2-RF is likely to contain one of two putative nuclear-targeting sequences (NTS) and a Zn²⁺ finger motif, but not the MCM box, encompassing Walker A/B ATPase motifs, evolutionarily conserved in the MCM2-7 family. For quantitative determination of MCM2 mRNA, TaqMan[®] Gene Expression Assays were carried out detecting transcripts derived from exonic boundaries of the MCM2 gene, indicated by open vertical bars. Note that the TaqMan[®] assay detecting the exon 3–4 overlaps the gene region encoding amino acids 131–150.

upon hTERT transduction (Fig. 2A). Interestingly, a novel band was induced reciprocally in senescing cells (Fig. 2A). This molecular mass of approximately 55 kDa was detected by an anti-MCM2 antibody raised against synthetic peptides corresponding to amino acid residues 131–150 of the MCM2 protein (Fig. 1), and thus designated as a MCM2-related fragment. Since there is no known protein with substantive similarity to this peptide sequence, we hypothesized that the MCM2-related fragment may represent a novel splicing variant or posttranslational cleavage of MCM2, and that its expression is associated with the status of cell proliferation.

To determine a possible splicing variant, quantitative PCR was performed by employing three independent sets of primers and a TaqMan[®] probe, in which the MCM2 exon 3–4 boundary region is specifically detected by the sequence encoding amino acids 131–150 of MCM2. Figure 2B demonstrates that all of the TaqMan[®] assays detected a reduction in MCM2 mRNA expression upon cellular senescence in parallel with the protein level. In addition, Northern blotting with a full length MCM2 cDNA probe detected a single transcript only throughout the PDs of EPC2 cells with or without hTERT expression (data not shown), although the sensitivity of detecting low copy mRNA species was not as great as that by PCR. These observations argue against the idea that a unique splicing variant is expressed upon cellular senescence.

MCM2-related protein fragment is also induced upon contact inhibition and terminal differentiation, but not apoptosis. We next tested whether or not the MCM2-related fragment may be induced by cellular conditions other than senescence and detected by other MCM2 antibodies raised against different epitopes within the amino-terminal or carboxyl-terminal regions (Fig. 1). First, EPC2 cells were grown in monolayer culture until they reach an overly confluent status. Contact inhibition was confirmed by decreased cell proliferation and pRB phosphorylation (Fig. 3B). As shown in Figure 3A, the MCM2-related fragment was detected progressively by only the anti-MCM2 antibody recognizing amino acid residues 131–150 in post confluent day 3 through day 11 while downregulation of the full length MCM2 protein was detected consistently by all of the antibodies in postconfluent cells (Fig. 3A). Thus, the MCM2-related

fragment was thought to lack both amino- and carboxyl-termini of the full length MCM2 protein as denoted in Figure 1.

Such an induction of the MCM2-related fragment was observed also in hTERT-immortalized EPC2 cells upon contact inhibition (Fig. 3C and data not shown). Of note, the MCM2-related fragment was expressed weakly in subconfluent EPC2 cells, where subpopulation cells undergoes spontaneous senescence in primary culture (Figs. 3A and 4A).

Next, EPC2 and EPC2-hTERT cells were treated with calcium chloride to induce terminal differentiation as indicated by induction of involucrin (Fig. 4). Again, the full length MCM2 was down-regulated and the MCM2-related fragment was induced, indicating that the MCM2-related fragment can be induced upon cell growth inhibition associated with cellular senescence, contact inhibition or terminal differentiation. Finally, cells were exposed to actinomycin D or gamma-irradiation. While apoptosis was induced within 12–24 hours as indicated by fragmentation of caspase 3, the MCM2-related fragment was not detected (Fig. 5 and data not shown). Importantly, the full length MCM2 level was not altered by apoptosis. These data indicate that the MCM2 protein undergoes a molecular shift from the full-length form to a short form upon chronic, but not acute, induction of cell growth inhibition or cell cycle arrest.

MCM2 protein expression may be regulated through post-translational modification. The above data suggested that the full length MCM2 protein may be cleaved to generate the MCM2-related fragment upon cell growth inhibition. However, the lack of both amino- and carboxyl-terminal regions (Fig. 1) precluded us from determining whether the MCM2-related fragment is derived from the full-length form of MCM2 protein. Nonetheless, we expressed ectopically an amino-terminally Flag-epitope tagged full-length MCM2 protein in EPC2-hTERT cells. Flag-tagged MCM2 was expressed constitutively under the retroviral LTR promoter. Interestingly, contact inhibition or terminal differentiation reduced the expression of the Flag-tagged MCM2 (Figs. 3C and 4B), suggesting that MCM2 protein may undergo degradation and/or cleavage although the resulting putative MCM2-related fragment per se could not be detected by an anti-Flag antibody.

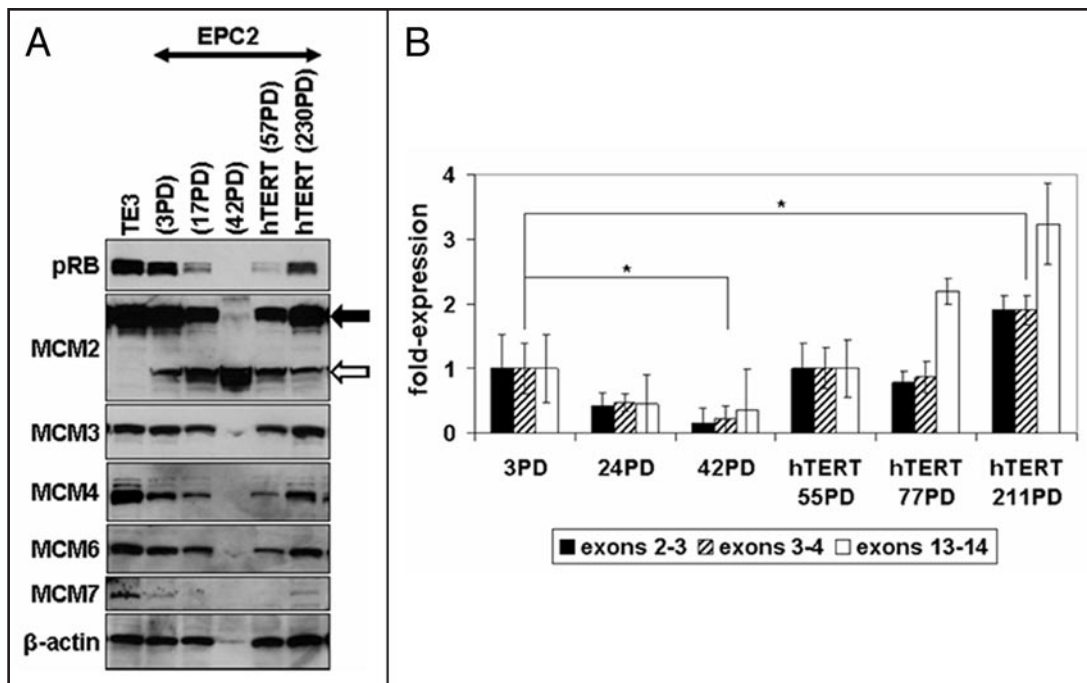


Figure 2. Reciprocal expression of MCM2 protein and the MCM2 fragment with senescence and immortalization of primary human esophageal keratinocytes. (A) Western blotting demonstrates progressive downregulation of examined MCM2-7 family proteins, including the 120 kDa MCM2 (solid arrow) in EPC2 cells undergoing replicative senescence by 42 population doublings (PD) while they were reinduced variably upon immortalization of EPC2 by hTERT. By contrast, an MCM2 fragment (open arrow) was reciprocally induced upon senescence and suppressed by immortalization when Western blot was carried out with an MCM2 antibody directed against amino acids 131–150 of MCM (Fig. 1). Cellular senescence was accompanied by a reduction in the phosphorylation level of RB protein. MCM2, but not the MCM2 fragment, was detected in TE3 esophageal cancer cells, an esophageal squamous cell carcinoma cell line. β -actin was used as a loading control. Note that protein yield from presenescent cells (42PD) was low and only 1 μ g of protein was loaded while other lanes were loaded with 10 μ g of protein. Nonetheless, the MCM2 fragment was detectable at 42PD. (B) MCM2 mRNA level was determined by TaqMan[®] Gene Expression Assays at indicated population doublings using a set of sequence specific primers and a probe detecting different exonic junctions of the MCM2 gene transcript as described in Figure 1.

In aggregate, the MCM2 protein may give rise to a putative cleaved form designated as a MCM2-related fragment, lacking both amino- and carboxyl-terminal regions as well as the MCM box of MCM2 (Fig. 1) upon cellular senescence, contact inhibition or terminal differentiation, but not apoptosis.

Discussion

MCM2-7 proteins are related to each other and form a complex for DNA synthesis initiation. The MCM complex binds origins of DNA replication during the late M/early G₁ phases of the cell cycle. Through the action of S phase protein kinases, the MCM complexes unwind the double-stranded DNA at the origin, with subsequent recruitment of DNA polymerases and initiation of DNA synthesis. They then disengage from replication origins, so DNA replication cannot be reinitiated. MCMs are targets of S phase checkpoints. That being said, in premalignant and malignant conditions, the MCMs are often expressed abnormally, and have been advocated as serving as diagnostic biomarkers to distinguish between normal cells and malignant cells, and in cancers themselves as potential therapeutic targets. In fact, we have observed marked upregulation of MCM2 mRNA and its 120-kDa protein product in primary esophageal tumor tissues as well as actively proliferating esophageal cell lines (Fig. 2 and unpublished observations). Nevertheless, by virtue of MCM dysregulation, chromosomal aberrations accrue during tumorigenesis; loss of MCM proteins triggers DNA damage and genomic instability.

Our data with MCM2 mRNA quantitation with three independent probe sets (Fig. 2B) did not support the possibility of de novo transcription of an alternative spliced form to account for the novel MCM2-related fragment. Although post-translational cleavage of MCM2 protein is suggested, neither the precise cleavage site(s) nor the responsible proteases were identified in the present study. First, *in silico* analysis using the peptide cutter program¹⁰ failed to identify promising cleavage sites for MCM2 compatible with the predicted cleavage pattern (Fig. 1) based upon the distribution of epitopes for the antibodies we used. Second, the lack of either amino- or carboxyl terminal region in the MCM2 related fragment hampered the use of epitope-tagged protein to be expressed ectopically. In fact, aminoterminally Flag-epitope tagged MCM2 only disappeared following differentiation (Fig. 3C). Finally, a low protein yield from the senescent or terminally differentiated cells limited affinity purification with an antibody. Amongst the MCM family members, MCM3 has been reported to undergo proteolytic cleavage mediated by Caspase-3.¹¹ Consistent with that notion, MCM2 remained uncleaved upon caspase-3 activation (Fig. 5).

Squamous epithelial cells, or keratinocytes, are distinguished by their proliferation-differentiation gradient and renewal during homeostasis and tissue regeneration. Such cells slough that is triggered by apoptosis and senescence prior to renewal. While the expression of MCMs is decreased, if not lost, during differentiation in somatic cells,¹² it is unclear to what extent changes in their structures and functions impact upon differentiation. We have discovered

that while all of the MCM family members were markedly downregulated during cellular senescence (Fig. 2) and differentiation (data not shown), there is the emergence of a unique fragmented MCM2 protein that may arise due to cleavage, and lacks both the amino- and carboxyl domains as well as the MCM box. It is conceivable that this fragment is responsible for preventing DNA replication during periods of prolonged or terminal differentiation, and/or during senescence. In a recent study, Komamura-Kohno Y, et al., analyzed the MCM2 protein biochemically through partial digestion with trypsin.¹³ They demonstrated that MCM2 fragments derived from the C-terminal region inhibit DNA helicase activity through their ability to bind to ssDNA. By contrast, two fragments (148–441 and 442–676) from the central region were responsible chiefly for the interaction between MCM2 and MCM4.¹³ Thus, the MCM2 fragment identified in our study may prevent assembly of the MCM2-7 complex. It is also quite possible that fragments of MCM2 might confer different effects in comparison to full-length MCM2, depending upon subcellular localization based upon solubility, or lack of it, as has been reported in mouse ovarian oocytes,¹⁴ implying there may be posttranslational modification of MCM2 in association with different functional properties.

Materials and Methods

Cell culture, retroviral vectors and retroviral transduction. Normal human esophageal epithelial cells (EPC2) and their hTERT-immortalized derivative (EPC2-hTERT) were described previously.⁷ A full-length human MCM2 cDNA was generated by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA extracted from EPC2 cells as a template. The PCR product, tagged with *Bam*HI and *Xho*I sites at the 5' and 3' ends, respectively, was cloned into the pCMV FLAG-tagging mammalian expression vector (Stratagene). For stable expression, FLAG-tagged human MCM2 coding sequence was further subcloned into the pBabe puromycin-resistant retrovirus vector.¹⁵ Retrovirus production and infection were carried out as described previously.^{7,16} In brief, retroviral expression vectors were transfected into Phoenix-Ampho packaging cells and the virus-containing culture medium supernatants were collected 48 and 72 hours post-transfection. EPC2-hTERT cells were infected with the virus in a 6-well plate (0.5×10^5 cells/well), followed by drug selection with $0.5 \mu\text{g/mL}$ of puromycin for 5 days.

RNA extraction, cDNA preparation and real-time PCR. Briefly, total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). DNaseI treatment was done on column and cDNA was synthesized with the SuperscriptTM First Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed using the ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), according to the manufacturer's

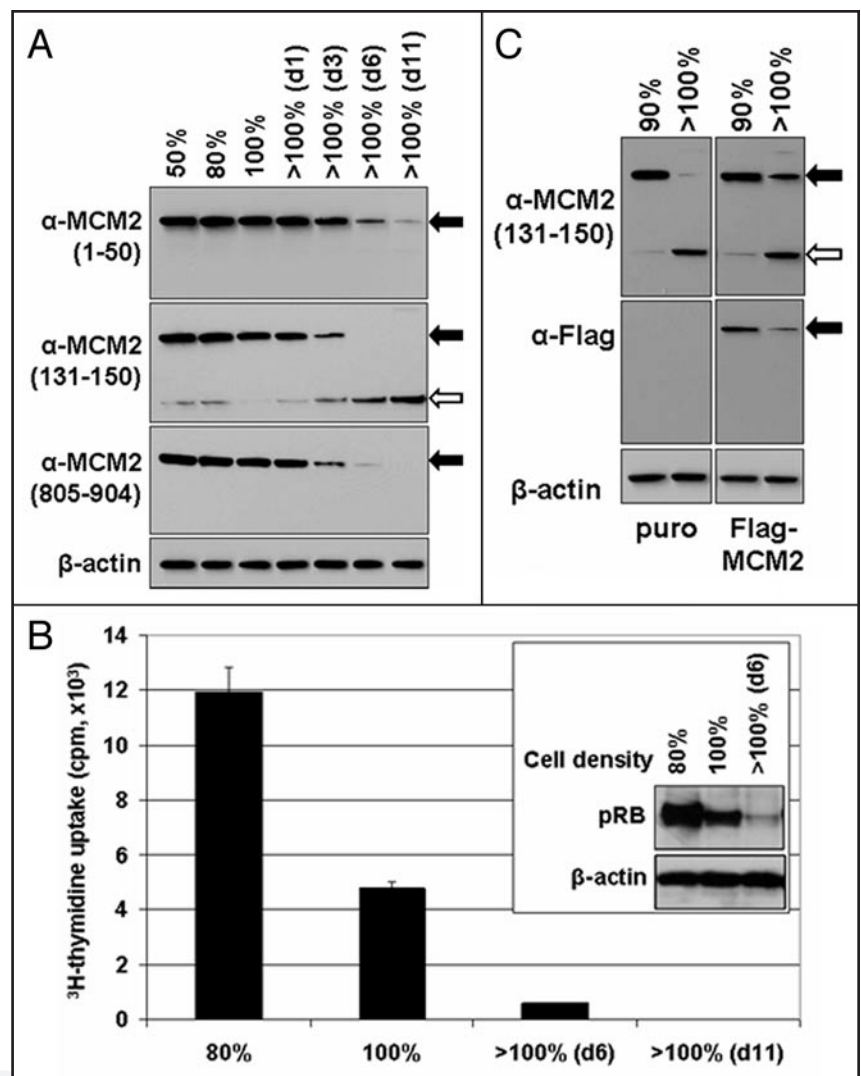


Figure 3. Contact inhibition of human primary esophageal keratinocytes is associated with reduction in the full-length MCM2 protein and induction of the MCM2 fragment through a posttranscriptional mechanism. (A) Western blotting using anti-MCM2 antibodies recognizing different epitopes as depicted in Figure 1 detects changes in expression of full-length MCM2 (solid arrow) and an MCM2-related fragment (open arrow) in EPC2 cells harvested at various cell densities. In (B), contact inhibition was documented by a reduction in cell proliferation. ³H-thymidine incorporation was adjusted by the amount of protein from the whole cell lysate used for Western blotting to detect the hyper-phosphorylated form of RB protein shown in the insert. In (A and B), days elapsed after 100% confluent status are indicated in the parentheses. (C) EPC2-hTERT cells stably transduced with amino-terminally Flag-epitope tagged MCM2 protein (Flag-MCM2) or a control empty vector (puro) were harvested at 90% cell density or 3 days after reaching 100% confluency and subjected to Western blotting with the indicated antibody. Note that anti-Flag antibody detected a reduced level of exogenously expressed epitope-tagged full-length MCM2 protein but not MCM2 protein that lacks the amino-terminal sequence (Fig. 1). β-actin was used as a loading control.

instructions. The MCM2 mRNA was determined by TaqMan[®] Gene Expression Assays (Applied Biosystems) using three independent sets of primers and the probes (Hs00170472_m1 for exons 2–3, Hs01091568_g1 for exons 3–4 and Hs01091564_m1 for exons 13–14), targeting different exon boundaries of the MCM2 transcript (MN_004526.2). SYBR green reagent (Applied Biosystems) was used to quantitate mRNA for GAPDH as an internal control. All PCR reactions were performed in triplicate. The relative expression level of MCM2 mRNA was calculated by normalizing to GAPDH

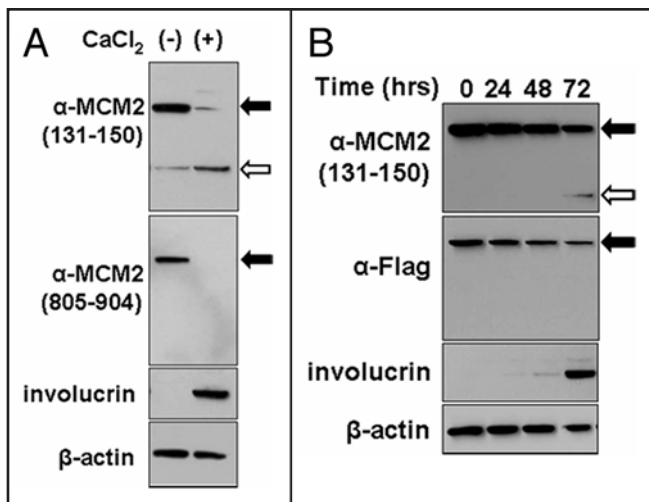


Figure 4. Terminal differentiation in primary human esophageal keratinocytes is associated with a reciprocal expression of MCM2 and the MCM2 fragment. Subconfluent EPC2 cells (A) and EPC2-hTERT cells stably expressing Flag-epitope tagged MCM2 (B) were treated for up to 72 hours with 1.8 mM CaCl_2 to induce terminal differentiation which was documented by Western blotting for involucrin. Indicated anti-MCM2 antibodies and anti-Flag antibody detected full size MCM2 (solid arrow) or a MCM2 fragment (open arrow). β -actin was used as a loading control.

mRNA expression level. Data were analyzed using ABI PRISM® 7000 sequence detection system software (Applied Biosystems).

Immunoprecipitation and western blot analysis. 10 μg protein from whole-cell extracts was separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membranes were incubated with anti-MCM2 (A300-191A) (amino acid residues 1–50) (Bethyl laboratories, Montgomery, TX), anti-MCM2 (559542) (amino acid residues 131–150) (BD Pharmingen), anti-MCM2 (clone 6A8) (amino acid residues 805–904) (Novus Biologicals, Littleton, CO), anti-MCM3 (BD Pharmingen), anti-MCM4 (BD Pharmingen), anti-MCM5 (BD Pharmingen), anti-MCM6 (BD Pharmingen), anti-MCM7 (BD Pharmingen), anti-FLAG M2 (Sigma), AC-74 against β -actin (Sigma), and DMA1A + DM1B against tubulin (Neo Markers). Staining was detected by using ECL Plus (Amersham Pharmacia biotech). The FLAG-tagged proteins were immunoprecipitated using FLAG Tagged Protein Immunoprecipitation Kit (Sigma).

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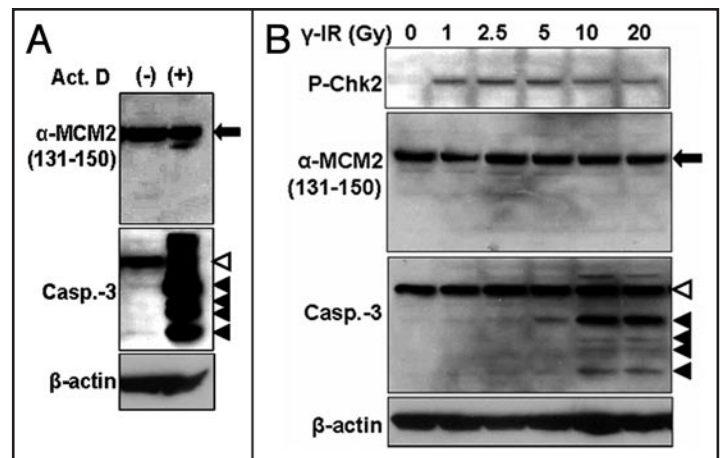


Figure 5. Caspase-3 activation does not affect MCM2 protein. EPC2-hTERT cells were treated with or without 2 $\mu\text{g}/\text{ml}$ actinomycin D (Act. D) for 5 hours (A) or exposed to indicated amount of γ -ray for 24 hours (B) and subjected to Western blotting to detect MCM2 (solid arrow), full-size (open triangle) or fragmented (solid triangle) forms of caspase-3 (Casp.-3), and Phospho-Chk2 (P-Chk2). β -actin was used as a loading control.

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